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A reliable method for estimating the postmortem interval from the biochemistry of the vitreous humor, temperature and body weight

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ABSTRACT

The estimation of the time elapsed since death is of paramount importance in the field of forensic sciences and criminal investigation, owing, among other factors, to the possible legal repercussions. Over the past few years various formulae have been developed to calculate this interval using a combination of different statistical methods and the concentrations of substances found in the vitreous humor. Corrective factors, such as ambient temperature, cause of death or age, which can modify the concentration of these substances and therefore the estimation of the postmortem interval, have been incorporated into models. In this paper five simple and reliable models to estimate PMI based the on the analysis of potassium, hypoxanthine and urea in the vitreous humor are presented. Corrective factors, such as body weight, rectal temperature and ambient temperature, which can influence the estimation of this interval have been incorporated into the formulae. Finally, the R^2 and the mean squared error have been calculated for each model in order to select the best of the five. A free software program which calculates the PMI from the model and parameters used is available from the authors. It provides quick and reliable results as well as the error committed and R^2 for each case.

1. Introduction

The estimation of the time elapsed between death and the finding of the cadaver, known as the postmortem interval (PMI), is of paramount importance in the field of forensic sciences and criminal investigation, owing, among other factors, to the possible legal repercussions [1–5]. Establishing PMI is one of the fundamental tasks of the forensic pathologist when a body is found [3]. Despite the numerous methods described, PMI estimation remains under study due to the lack of a validated method that is assumed to be accurate and universal.

While some methods show a quantified precision of estimation and consider the influence of possible modifying factors, such as methods based on supravital phenomena [4], others derive from empirical conclusions based only on experience, such as the study of gastric contents [5].

While there are numerous methods for estimating time since death, the most widely used methodology for estimating PMI is based on cadaveric cooling measured by rectal temperature [5–16]. However, it is known that the cooling rate of the corpse is influenced by environmental and particular conditions, such as ambient temperature, body weight or situations of hyper and hypothermia ante mortem. In this sense different nomograms have been proposed to estimate PMI by means of cadaveric cooling, which basically take into account weight, rectal temperature and ambient temperature [16]. In addition, this method should only be used while the body temperature is not yet equilibrated with the ambient temperature [16] and it is limited to environmental factors or circumstances of death [7].

Over the last 50 years, and in the wake of technological development, chemical methods have also been proposed to

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estimate PMI. The first studies were performed on blood [8] and cerebrospinal fluid [9], and above all on the vitreous humor (VH) [10,11], probably because autolytic changes occur more slowly due to its being anatomically protected and isolated by its own structure and cranial bones, so that it could be studied even after severe head trauma. Moreover, it is less subject to contamination and putrefaction than other biological matrices [12], and the slower rate of chemical change extends the period of time for PMI estimation [12,13].

In recent years, various studies have demonstrated the usefulness of the vitreous humor to estimate PMI in recent cadavers by determining the levels of potassium (K^+) and hypoxanthine (Hx) [5,14–18]. In those cases, the most commonly used techniques are high performance liquid chromatography (HPLC) [10,15,18–20] and liquid chromatography coupled to tandem mass spectrometry (LC-MSMS) [21,22] and for K^+ the usual method involves using an indirect potentiometry-specific electrode [19,23–24].

Despite the numerous studies done in this field, there is still no consensus as to what formula or method is most accurate to estimate PMI from increased potassium and hypoxanthine in VH [23]. The concentrations of both substances increase as PMI increases [14–18]. There are certain conditions that affect the methodology: correct extraction of VH, pre-treatment, ambient temperature, body weight and temperature, previous metabolic status, agony preceding death, and even the cause of death are factors that can modify the results of PMI estimation [12,21,25–28]. Based on this fact, recent methods have been designed to determine the PMI based on the biochemistry of the VH as well as correctional factors such as ambient temperature and age, with apparently good results [23,29]. Nevertheless, it is essential to continue research in this field and to develop new methodologies that enable this calculation when the necessary data to apply existing methods is not available, as well as improving the predictive power of these models.

The availability of different methods of validation that integrate different parameters makes their use in daily practice more feasible and the option to choose one method or another based on the available data and the particular circumstances of each case helps to establish PMI in a more reliable way.

In order to develop a methodology capable of estimating PMI with a narrow margin of error, we have designed and validated different simple models that integrate the analytical values of VH, ambient temperature (*Tamb*), rectal temperature (*Trectal*) and the weight (*Weight*) of the corpse. All of these variables are easily obtained and quickly measured.

2. Material and methods

We examined 331 vitreous humor samples from cadavers of forensic interest, all of which had suffered sudden and unexpected death (natural or violent) and were received for autopsy at the National Institute of Forensic Medicine and Forensic Sciences, Centro-Coimbra delegation (Portugal). These samples were taken from corpses whose autopsies revealed no indications of chronic disease, hyper or hypothermia, with a known postmortem interval of ±15 min after the police and/or judicial investigation.

The eyes of all corpses were closed when the samples were taken. A database was drawn up stating the sex, age, weight, postmortem interval rectal temperature, ambient temperature at the time of sampling, cause of death and whether or not resuscitation maneuvers had been performed, as well as the concentration of potassium (K^+), hypoxanthine (*Hx*), xanthine (*X*), guanine (*G*), sodium (Na^+), urea (*U*) and uric acid (*UA*). All cadavers were weighed naked on the same hospital scale. Sampling was performed by scleral puncture near the external cantus, using a 10 ml syringe and a 20 gauge needle. Virtually all of the VH was removed. Parameters were determined twice and the mean value was used.

2.1. Determination of potassium and sodium

An Advia 2400 from Siemens was used for the analytical determination of K^+ and Na^+. This equipment is based on the indirect potentiometry method, which uses a selective electrode for the different ions (ISE). It is a method of habitual use in central hospital services for determination of electrolytes in forensic science [23,31,32] and it is periodically validated for quality purposes: once with internal controls every 60 analyzed samples and once a month with external controls.

2.2. Hx, X, G, U and UA determination

The determination of Hx, X, G and UA of VH was performed by LC-MSMS, following the methodology described by Lendoiro et al. [22]. Centrifugation was used as analytical pretreatment, as Camba et al. [12]. Table 1 shows the chemicals and reagents used.

The samples were centrifuged for 10 min at 14,500 rpm in a Mini Spin Plus (Eppendorf, Hamburg, Germany). Two mL of 2 mM ammonium hydroxide and 25 μL of internal standards (IS) were added to an aliquot of 150 μL of vitreous humour.

Solid phase extraction (SPE) using OASIS MAX cartridges (Waters, Mildford, USA) was employed for sample clean-up. Cartridges were conditioned with 2 mL of methanol and 2 mL of water before loading the samples into the SPE column. Then, sequential washes were performed with 2 mL 5% ammonium hydroxide in water and 2 mL 5% ammonium hydroxide in methanol. After drying the cartridges for 10 min under vacuum, elution was performed in two steps: first with 2 mL of 2% formic acid in water and second with 2.5 mL of formic acid in methanol. The eluates were collected in the same tube and evaporated with nitrogen at 40 °C. Finally, the samples were reconstituted in 100 μL of 10 mM ammonium acetate (pH = 4.5), and 30 μL were injected into LC-MSMS.

A Waters Alliance 2795 HPLC Separation Module with a Waters Alliance series column heater/cooler (Waters Corp, Milford, USA)

Table 1
Chemicals and reagents used.

Chemical/reagents	Commercial supplier	Provenance
Hx, G, X, UA (solid form)	Acors Organics	Geel, Belgium
internal pattern (5-(p-methylphenyl)-5-phenylhidantoin)	EGA-Chemie	Steinheim, Germany
acetonitrile, ammonium acetate, metanol	Panreac Quimica S.A.U	Barcelona, Spain
amonium 30%, Formic acid	Scharlau	Spain, Sentmenat
OASIS MAX cartridges (60 mg, 3 mL)	Waters	Mildford, USA
hyaluronidase (bovine testes)	Sigma Aldrich Chemie	USA
purified water	Milli-Q system	Le Mont-sur-Lausanne, Switzerland

was used for the analysis of the samples. Chromatographic separation was performed using an analytical column Atlantis T3 (2.1 mm × 100 mm, 3 μm), working in gradient mode, with acetonitrile and 10 mM ammonium acetate (pH 4.5) as mobile phase. For detection, a Quattro Micro tandem mass spectrometer (Waters, Mildford, USA) was used working with electrospray in positive mode (ESI+) and multiple reaction monitoring (MRM) mode. Two product-precursor transitions were monitored per compound, except for IS, for which only one transition was selected. Data acquisition was controlled using MassLynx 4.0 software and processed with QuanLynx 4.0 software (Waters Corp, Milford, USA).

The method was subjected to a complete validation, including linearity, limit of detection (LOD), limit of quantification (LOQ), imprecision, analytical recovery, extraction efficiency, process efficiency and matrix effect following the methodology presented by Lendoiro et al. [22].

In this study we have employed the best available techniques for determining the measurements of the variables involved taking into account that prediction models inherit the good/bad properties (accuracy, significance, relevance, . . .) of the measurements. This can be seen in the validation stage analyzing some goodness-of-fit indicators (prediction errors, coefficient of determination, residual variance . . .).

2.3. Evaluation of blood contamination

Accidental puncture of the blood vessels of the eye during VH extraction may alter Hx concentration values because the samples are contaminated with blood leading to erroneous estimates of PMI [10,15,22]. Applying the methodology presented by Lendoiro et al. [22], MRM transitions of the analytes of the UA were analyzed and only those samples that did not present blood contamination were included in the study (331 samples).

2.4. Statistical method

Statistical analysis of the data was carried out using the statistical package R [33]. The models were designed using the Generalized Additive Models (GAM) [34], obtained from the package mgcv available at http://cran.es.rproject.org/web/packages. These models are usually used as an extension of traditional linear models, especially when there are continuous covariates [35].

GAM models are more general that linear ones in the sense that they can represent quadratic, cubic or smooth contributions of the covariates instead of simple linear addings [34,35].

Additive models are commonly used in forensic science, not only for estimating the postmortem interval [36], but also for estimating other conditions such as stature [37].

Once the models were designed, a comparison was made using a usual cross-validation strategy to establish their predictive capacity in an impartial way. The sample was randomly separated into two groups (Training: 80%/Validation: 20%). The data from the first group are used for the estimation of the models, and then applied to the remaining sample to obtain predictions for data not used in the estimation step. This process was randomly carried out 1000 times and finally, the mean squared error of all replications was computed.

Finally, we estimate the PMI of our cases by using the Henssge's Nomogram [16,30], frequently used in forensic science, in order to compare its mean squared error with those obtained with our methodology.

The study would not need a specific permit, since all the analyzed substances used as variables in the study are determined routinely in the investigation of a suspicious or violent death, they were not determined specifically for this work, and do not pose any risk to the person or the criminal investigation. In addition, the data used are anonymized, thereby protecting the duty of secrecy. Notwithstanding the foregoing, it was approved by the Clinical Research Ethics Committee of Galicia, 2009/390.

3. Results

The descriptive analysis of the variables used to perform this study is shown in Table 2.

One by one, *Trectal, Hx, K^+* showed some nonlinear relationship with PMI. This can be clearly seen in Fig. 1 where an estimate of the relationship among these variates and PMI is shown. Additionally, *Tamb*, *U* and *Weight* condition these relationships. That is why five different models were taken for data analysis using those variables that produced some effect on PMI: *Trectal, Tamb, Hx, K^+, U* and *Weight*.

On studying the data, we observed heteroscedasticity and high asymmetry to the right of the response variable (PMI) (Fig. 2), and therefore chose to transform this variable by logarithms. This is the usual trick in statistical models to treat those variables whose variance grows with its mean value as is the case of PMI. In making this transformation it was observed that the contribution of *Hx* is not purely linear, so the models were accordingly modified to allow greater flexibility.

Another indication that this transformation is necessary can be observed when comparing the PMI boxplots with *Trectal* (bottom left subgraph, Fig. 2), which are significantly wider for low *Trectal* values and narrower for high *Trectal* values. This is an evidence that it cannot be assumed that the variance of the PMI variable is constant. The effect is noticeably attenuated when it is transformed by logarithms as seen in the boxplot graph (bottom right subgraph, Fig. 2). This is an important consideration in many regression models (as is the case of linear models or in this case the GAM) since one of the usual hypotheses of these models is that the response variable is homoscedastic, that is, that the variance of the variable is constant and does not increase or decrease according to the covariates used. This hypothesis of homoscedasticity is used decisively in the construction of prediction intervals (which basically depends on the estimation of this variance and the

Table 2
Description of the variables.

	Age	Weight (kg)	PMI (h)	Tamb (°C)	Trectal (°C)	Hx (μmol/L)	K^+ (mmol/L)	U (mg/dl)
Min	18.00	35.50	1.50	9.30	22.20	10.20	4.60	12.00
1st Qu	46.00	62.83	4.00	14.47	31.20	42.95	6.00	27.00
Median	60.00	74.00	6.17	18.55	33.50	59.90	6.60	36.00
Mean	59.73	75.20	6.81	18.54	32.93	70.53	6.86	42.39
3rd Qu	75.00	87.50	8.25	22.40	35.12	93.53	7.40	46.00
Max	97.00	127.10	22.50	29.40	37.80	255.20	13.60	231.00
Na's/other	0	0	0	0	0	64	11	19

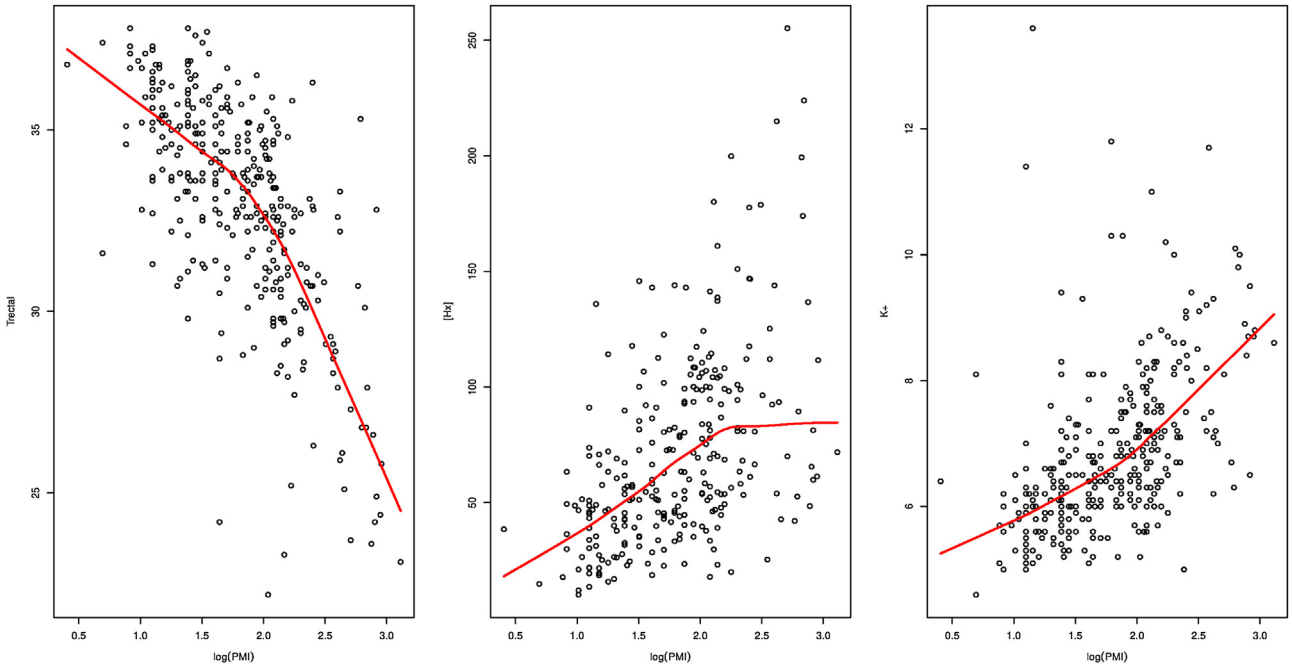

Fig. 1. From left to right, relationship between *Trectal*,*Hx* and K^+ with respect to log(PMI). In each case, red line is the lowess curve. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

distance of the covariates to their means). Therefore, in this type of modeling, it is mandatory to first transform the response variable to achieve homoscedasticity before applying the regression models. Once the prediction models have been applied, the transformation by logarithms can be easily reversed returning to the original scale of the response variable since this transformation is monotonous.

This reverse transformation (using exponentials) can also be applied to prediction intervals but losing their symmetry and changing their length depending on which side of the mean of the covariate is computed, as opposed to the homoscedastic model where the length depends only on the amount of deviation respect to the mean but not respect to its sign.

Five models were designed with the previously described variables where in each case α is the constant, f_i the partial effect of the i variable and ε the homoscedastic zero mean errors. So, all standard errors shown are in log-scale.

3.1. Model 1

$$\log(PMI) = \alpha + f_1(Trectal) + f_2(Hx) + f_3(K^+) + f_4(U) + f_5(Weight) + \varepsilon$$

This model integrates the variables *Trectal*, *Hx*, K^+, *U* and *Weight* and shows a standard error of 0.2391 and all variables obtained a statistically significant *p*-value ($p < 0.05$). Fig. 3 shows the effect of each variable in the model which basically are the expected ones. The amount of the effect can be deduced from the range of variation of the curves respect to the scale. The biggest effect is due to *Trectal* with a clear decreasing linear relationship with log(*PMI*). The response also grows as K^+ increases up to a value of 10 where a decreasing effect for higher values of K^+ is shown. The effect associated to *Hx* is similar (although smaller) but in this case when the value 150 is reached it is almost constant. The effects of *U* and *Weight* are only relevant for extreme values of both variables (in the case of *U* only for the highest values) suggesting that these variables are only needed to explain the PMI in border situations.

3.2. Model 2

$$\log(PMI) = \alpha + f_1(Trectal) + f_2(Hx) + f_3(K^+) + \varepsilon$$

With only three variables (*Trectal*, *Hx* and K^+), the model has a standard error of 0.2801 and all variables obtain a statistically significant *p*-value ($p < 0.05$). Fig. 4 shows the effect of each variable that integrates the model.

The effects of the covariates included in this model are basically the same as in model 1 but now, the effects of *Trectal* and *Hx* are trying to cover the lack of information provided in the previous model by *U* and *Weight* and so, change a little bit for lower values of *Trectal* and for the higher values of *Hx*.

3.3. Model 3

$$\log(PMI) = \alpha + f_1(Trectal) + f_2(Tamb) + f_3(Hx) + f_4(K^+) + f_5(U) + f_6(Weight) + \varepsilon$$

With respect to the first model, this one adds the *Tamb* that it is not significant maintaining the same level of standard error (0.2381). Fig. 5 shows the effect of each variable and clearly shows that the effect of *Tamb* is almost flat (second plot in the first row). The other effects are similar to those in model 1.

3.4. Model 4

$$\log(PMI) = \alpha + f_1(Trectal) + f_2(Tamb) + f_3(Hx) + f_4(K^+) + f_5(U) + \varepsilon$$

This model removes *Weight* from model 3 with a similar standard error (0.2607) and again, all variables, except *Tamb*, have a statistically significant *p*-value ($p < 0.05$). Fig. 6 shows the effect of

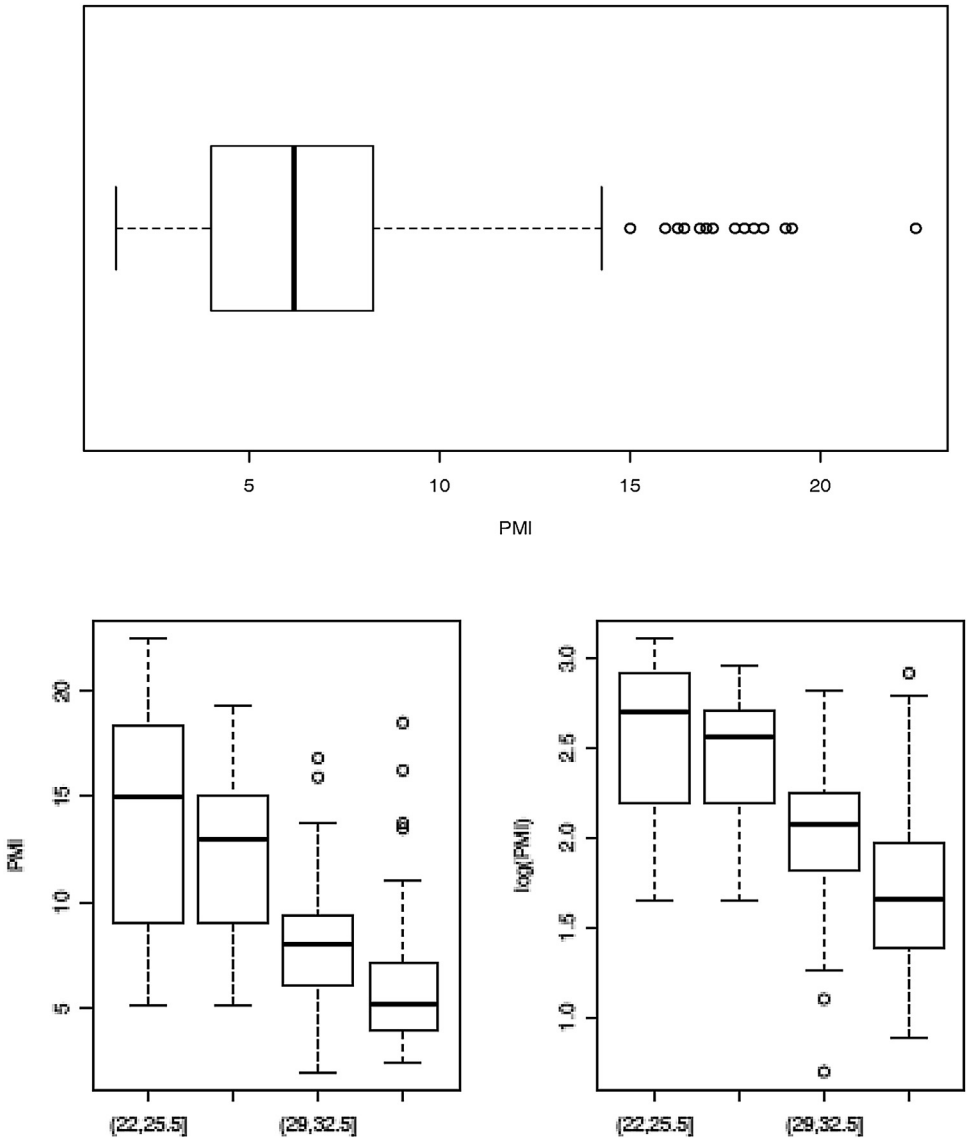

Fig. 2. PMI Boxplots. Top row: Boxplot of PMI. Bottom row: Comparison of boxplots for PMI and log (PMI), axis y, with respect to Trectal groups, axis x.

each variable that integrates the model. Comparing this model with Model 1, it is clear that the addition of *Tamb* cannot fill the hole (in terms of information) left by *Weight*.

3.5. Model 5

$$\log(PMI) = \alpha + f_1(Trectal) + f_2(Tamb) + f_3(Weight) + \varepsilon$$

This model has also been constructed using the most classic variables found in the literature [16], to be able to compare it with the classic Henssge's Nomogram method in terms of prediction accuracy. The model has a standard error of 0.3406. It follows the approach of the previous models in that the response variable has been transformed by logarithms and the contribution of the variables to the response has been freed to become smooth functions. For this model, apart from the individual contributions of the variables (Fig. 7), the response surface is shown by combining two variables (Fig. 8).

The effects of *Tamb* and *Weight* become now more significant in order to compensate for the gap in information left by K^+ and *Hx*. The joint effect shown in Fig. 8 suggests that for the same value of *Trectal*, the log(*PMI*) increases as the *Weight* increases. This can be an effect of the correlation among *Weight* and *Trectal* considering that the thermic equilibrium of the *Trectal* (when *Trectal* becomes equal to *Tamb*) is reached faster when the volume of the body is smaller (and so is the *Weight*).

Figs. 9 and 10 show the response surfaces for the PMI combining *Trectal* and *Hx* and *Trectal* and K^+ for the first four models. These three variables are the most significant ones in the models where they are included. These surfaces should not be used as direct application rules for models with three or more covariates since to construct the surface it is assumed that the covariates not shown are set using their mean value which, and due to the relationships between variables, is unreliable. These surfaces must be taken into account only as tools to describe the influence of the two variables shown according to the model. Likewise, and since the four models are explaining the same phenomenon, it is expected that the appearance of the four graphs is similar although here, Model 2 (which does not take into account the *Weight*) shows different patterns than the other three.

Finally, the cross-validation technique described above was performed and the mean square error of the estimate was

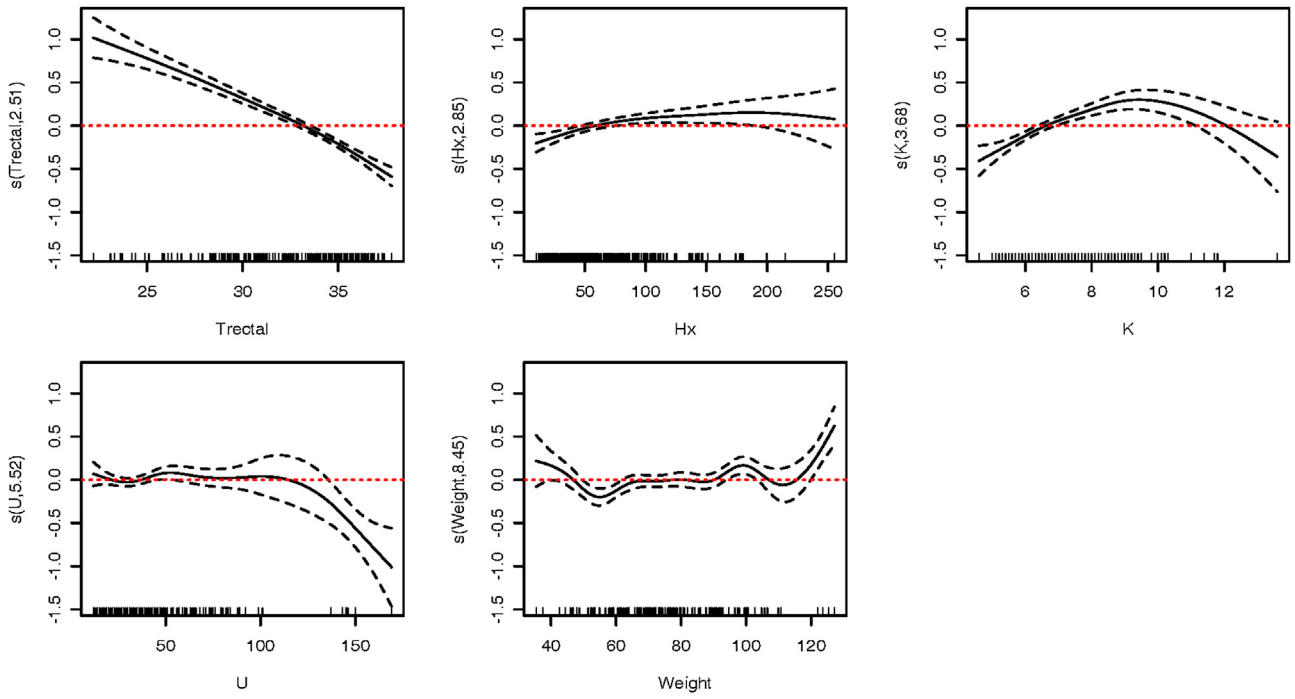

Fig. 3. Contribution of each variable that integrates model 1 on log(PMI). In order to interpret the effects, pay attention to the intervals where data are more dense. Red line represents no significant effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

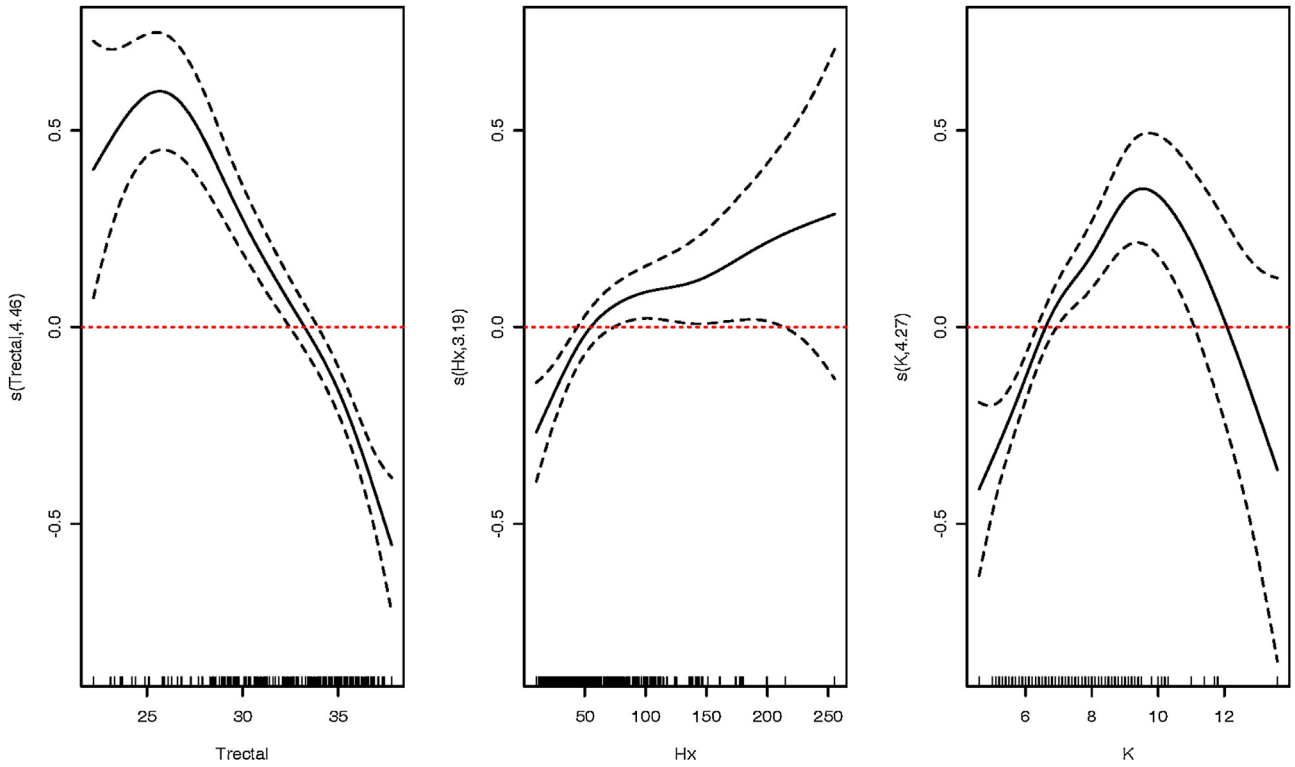

Fig. 4. Contribution of each variable that integrate model 2 on log(PMI). In order to interpret the effects, pay attention to the intervals where data are more dense. Red line represents no significant effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

calculated. The results obtained are shown in Table 3. This Monte Carlo procedure tries to evaluate the performance of the models under a practitioner's scenario in which we must predict new cases not included in the estimation step.

In fact, here the best model from the estimation perspective is Model 3, which maximizes the determination coefficient, R^2. From the prediction point of view, the best model is Model 1, which minimizes the MSE. This is a good example where the

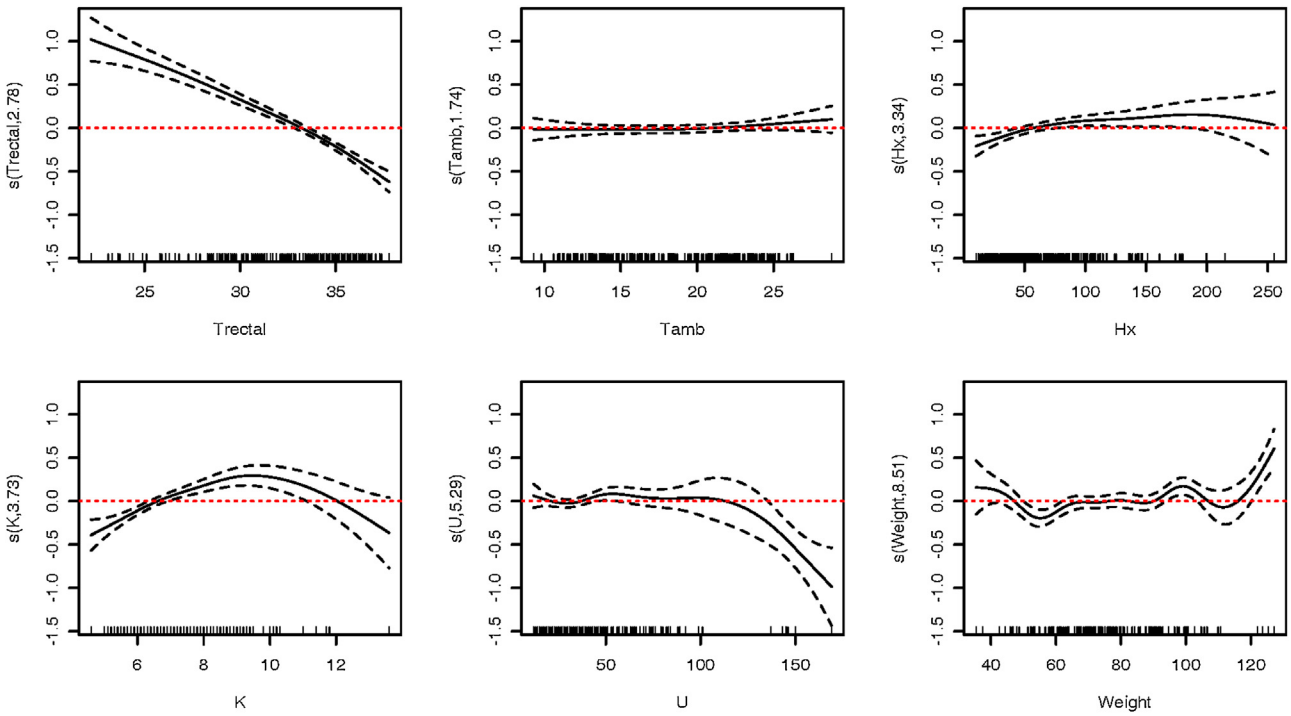

Fig. 5. Contribution of each variable that integrate model 3 on log(PMI). In order to interpret the effects, pay attention to the intervals where data are more dense. Red line represents no significant effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

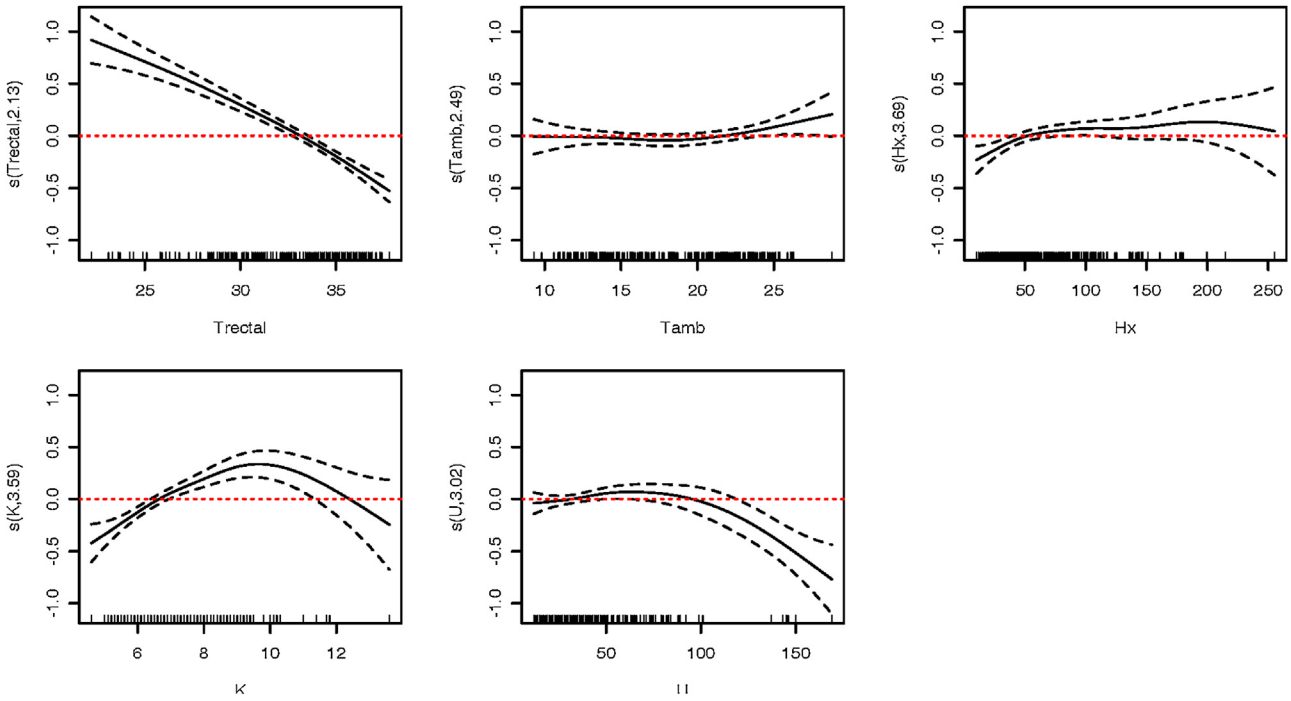

Fig. 6. Contribution of each variable that integrate model 4 on log(PMI). In order to interpret the effects, pay attention to the intervals where data are more dense. Red line represents no significant effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

addition of irrelevant variables to the model can lead to worse predictive models.

4. Discussion

The methodology most frequently used to estimate time of death is based on cadaverous cooling, or algor mortis [6], by means of the central temperature. However, the cooling rate of a corpse is influenced by environmental and individual conditions, such as clothing, the surface on which it is lain (grass, soil, upholstered) and air currents. To address these gaps, Henssge offers a series of empirical correction factors for body weight [38], as well as a tool for estimating PMI, known as Henssge's Nomogram.

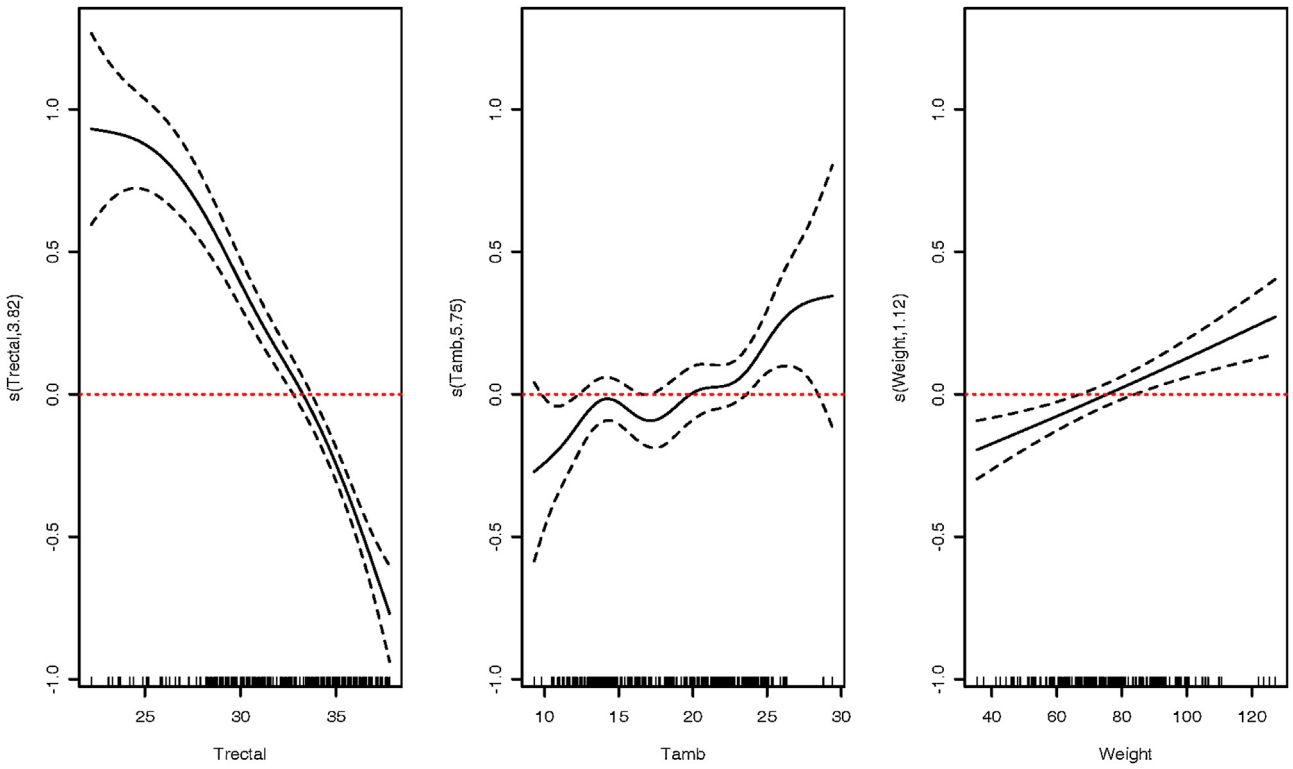

Fig. 7. Contribution of each variable that integrate model 5 on log(PMI). In order to interpret the effects, pay attention to the intervals where data are more dense. Red line represents no significant effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

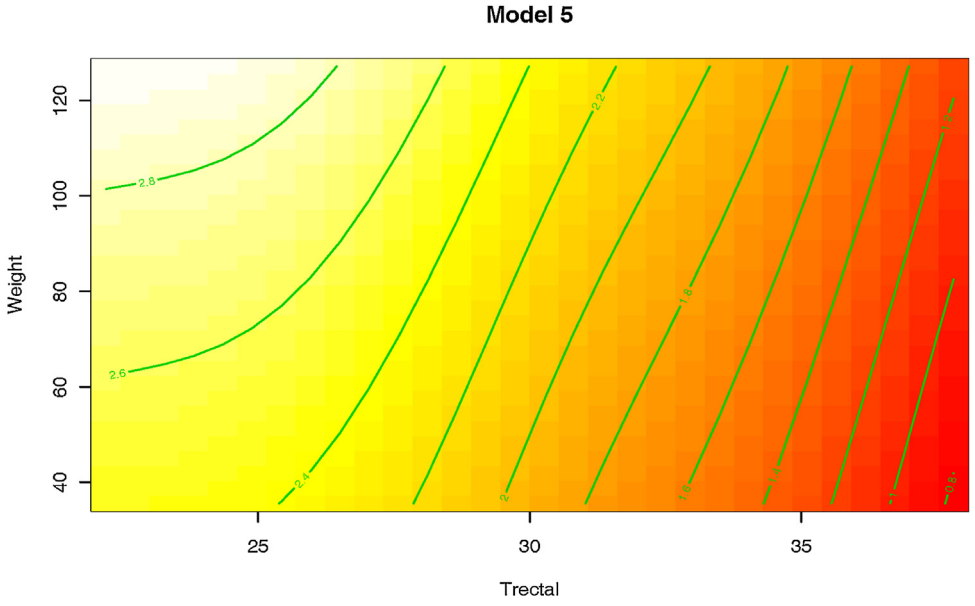

Fig. 8. Response surface combining *Trectal* and *Weight* for model 5.

Although this nomogram is widely used today, certain obstacles have been described that prevent or diminish its effectiveness in use, such as not knowing the ambient temperature at the time of death, cause of death (its use is not recommended in case of multiple injuries, death by burning or submersion [7]), sudden changes in environmental conditions between death and the discovery of the corpse and enormous subjectivity at the time of choosing the correction factor. In addition, this method should only be used while the temperature of the corpse is not yet equilibrated with room temperature and only offers a single confidence interval throughout the PMI. For these reasons, in recent years, the studies to determine the PMI have focused on physico-chemical models, especially in the VH biochemistry.

The study of the relationship between the concentrations of the different VH substances (mainly K^+ and Hx) has intensified and several studies using different conditions as correctional factors to minimize the error of estimation have been carried out.

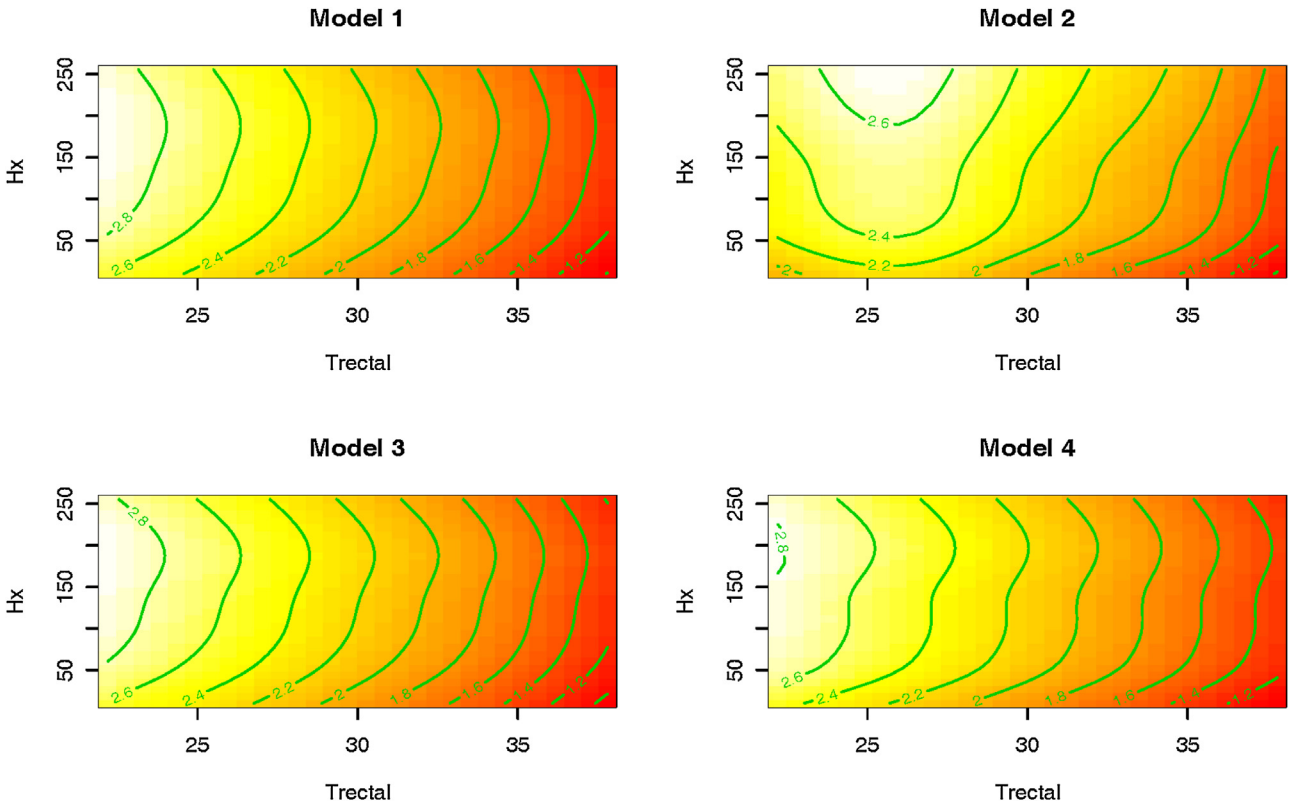

Fig. 9. Response surfaces combining *Trectal* and *Hx* for first four models. In this case, the rest of the variables of each model are fixed to their means.

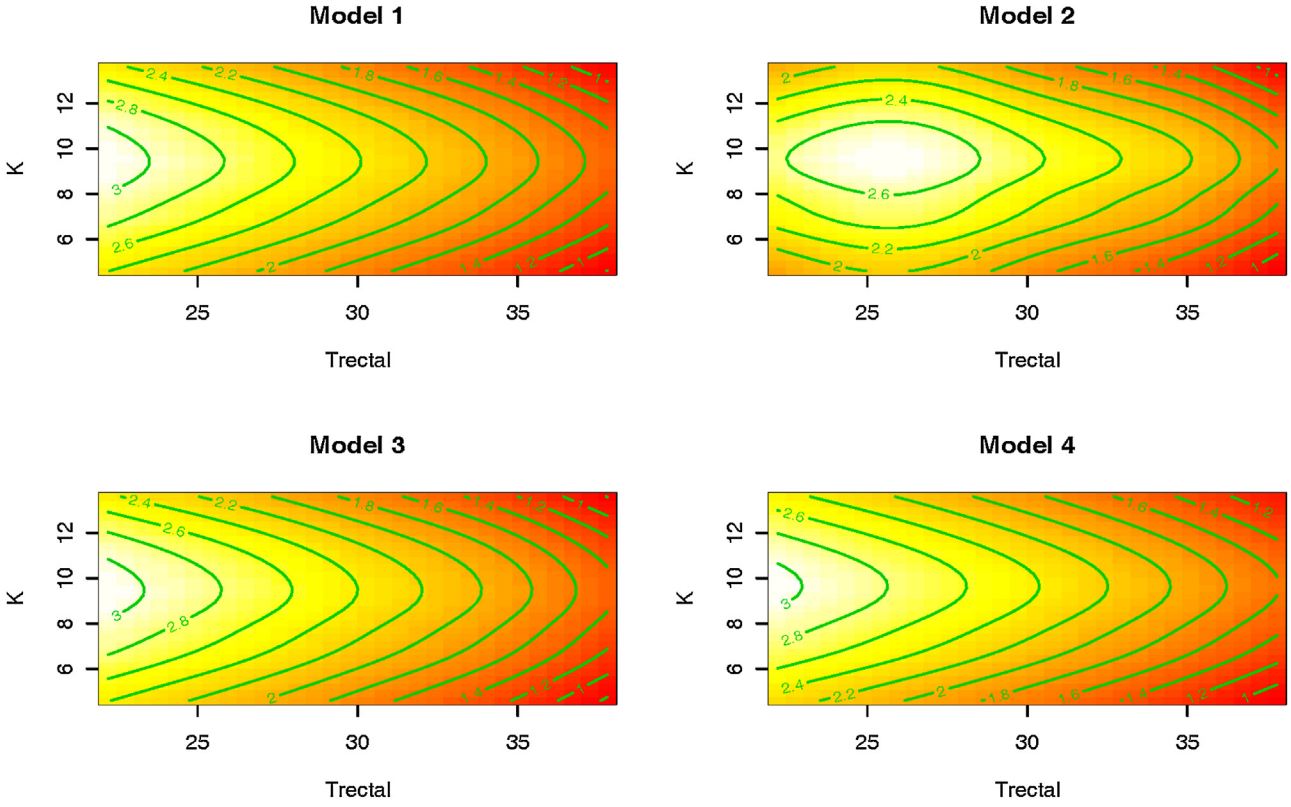

Fig. 10. Response surfaces combining *Trectal* and K^+ for first four models.

Table 3
A summary of the proposed models including their R^2 and quadratic prediction error (in logarithmic scale).

Variables	Trectal	Tamb	Hx	K+	U	Weight	R^2	MSE
Model 1	•		•	•	•	•	0.784	0.074
Model 2	•		•	•			0.702	0.088
Model 3	•	•	•	•	•	•	0.786	0.077
Model 4	•	•	•	•	•		0.743	0.080
Model 5	•	•				•	0.529	0.128
Henssge's Nomogram	•	•				•	—	0.215

In this paper, we present five methods developed from additive models, which take into account different significant factors for the estimation of PMI. The use of additive models is justified since these have shown greater predictive capacity in this context than linear regression models and have already been used in this field [21]. For this purpose, a sufficiently large number of human samples, 331, has been used to allow validation. The sample size is one of the largest published in this regard. There is great disparity in the origin and size of the samples used in the different studies that have been carried out in recent years [3,7,23,25,29,31]. We consider the use of a representative number of samples from individuals who suffered a rapid death with a minimum period of agony, in reality the majority of the so-called corpses of forensic interest [20], is necessary in order to avoid possible electrolytic alterations that could affect the VH and, and subsequently to an error in the estimation of PMI. Only in this way will it be possible to extrapolate the data obtained from corpses found in different out-of-hospital scenarios, an area where the need to estimate the time of death is vital. Not all the initial variables showed some relationship with the PMI. However, some did, although not linear, or they modified the relationship that existed between some of them and the PMI. The selection of the studied variables was based on previous studies that showed some relation with the PMI, mainly in linear models. Thus, we included the study of the U because it can modify the relationship between the potassium concentration and the PMI, the urea concentration remains stable throughout the PMI and it would indicate the electrolytic ante mortem state [15,39]. The Weight was used because it modified the relationship between Trectal and PMI according to [16]. The variables that were finally taken into account for the development of the models were those that were shown to have a statistically significant effect on the prediction of PMI, namely: T*rectal* and T*amb, Weight* according to [16] and *K*$^+$*, Hx, U* and *UA* in VH according to [12,15,22]. Neither the cause of death, unlike [20] and possibly because the additive methods make an estimate taking into account the joint effect of all the variables, nor resuscitation manoeuvers, nor age, eye (no differences between left or right eye) or sex and other analytes [20,21], showed statistically significant effects in this prediction. In our study only samples of clear appearance were used, and the presence or absence of UA [22] was checked as a control to discard samples contaminated with blood, since the impossibility of determining this in the VH sample, using the usual methods, indicates blood contamination. The presence of erythrocytes in the VH samples may alter the results of the estimation, so these samples were accordingly excluded.

Previous studies performed on both humans and animals have studied the influence of ambient temperature on potassium concentration in VH. Sturner and Gantner [1], Lie [40] and Adelson et al. [28] found that this has no influence on potassium concentrations in the vitreous humor, while other authors, such as Komura and Oshiro [41], Schoning and Strafuss [42] and McLaughlin [43], find that the increase in potassium in the vitreous humor is slower at lower temperatures, which agrees with the claim that biological processes slow down at low temperatures [23], in accordance with Van't Hoff's law, which states that the rate of chemical reactions are increased two or more times by each increase of 10° C in temperature [44]

The results of Zilg et al. [23] and Rognum et al. [29] also indicate an increase in the concentration of K^+ in response to a rise in ambient temperature. These authors consider ambient temperature as a correcting factor to enhance the accuracy of the models when estimating the postmortem interval. In our study, we have observed that using this parameter slightly helps to explain the variability of the data as a whole. While it is true that mathematical formulae have been developed to calculate ambient temperature [23], our own experience has been one of difficulty in reliably estimating the temperature fluctuations that a cadaver has been subjected to before its finding. Integrating the rectal temperature and body weight into the model can help solve this situation, since the body will cool more or less rapidly according to its body mass and the ambient temperature, as recorded in Henssge's Nomogram [30].

Zilg et al. [23] found no relationship between cadaver weight and increased K^+ concentration in VH, however, these authors did not relate this condition to PMI. The results of our study show that there is a relationship between body mass and the postmortem interval, and this statistically significant variable integrates in all models. It improves prediction results by increasing the explanation of the variability of the data and reducing the average square error committed when applying the model.

Another of the modulating factors is that of age. Previous studies have found that the age of the deceased influences the estimation of PMI [23], and, according to the data of this study, younger subjects present a higher postmortem increase in K^+ concentration, which could be explained by a smaller ocular diameter in younger individuals [10], although later studies have not been able to confirm this claim [45]. The results of our study show no influence of age in PMI estimation, although our data differ in age range and PMI studied.

The influence of age on the increase in the concentration of K^+ postmortem becomes more apparent, according to the Zilg study [24] at early ages. Our study focuses on an age range from 18 to 97 years and PMI on the first day, where age seems to have no influence on this parameter.

Although all the models designed and proposed in this paper offer a good explanation of the variability and a low average square error, the one that best adjusts the data, with an R^2 of 0.786, is Model 3, which integrates the variables *Trectal, Hx, K+, U*, weight and *Tamb*, although this is not the best predictive model. This is the typical effect of including non-significant variables in the model. In terms of predictive errors, the winner is Model 1, which has the advantage over Model 3 of being easier to apply, since it does not depend on ambient temperature. The variable *Tamb*, although very easy to measure, has the drawback that it is registered at a specific time and therefore does not take into account its route since death, which could yield more information about the PMI. This may explain its lack of relevant contribution in most models. Finally, model 5, constructed with the basic variables used in the literature, presents a clearly lower explanation than the other models ($R^2 = 0.529$) and a minimum squared prediction error that practically doubles the best of the other models (Table 3).

The results obtained in this study corroborate our previous hypotheses. The weight of the corpse has a very important effect on its cooling speed (Fig. 8) and, therefore, it is crucial to take it into account for the correct calculation of the PMI. In the same way, the analytical values of the *Hx* and *K*$^+$ present in the VH are intimately related to the PMI. The Fig. 3 shows how the effect of these substances increases when the PMI is increased, especially in the first hours. As the PMI lengthens, this relationship fades, possibly due to the beginning of putrefaction, or to a smaller number of cases in our sample.

There is no doubt that the greatest difficulty of the above methods, and those recently published, is their inability, once the possible variables have been determined, to provide a quick and easy calculation of the PMI. At this moment, we have developed a shiny application (shiny.rstudio.com) for internal use but in order to bring these models closer to daily forensic practice and to enable estimation of the PMI, we are planning to offer a web application from a server of our forensic institute.

The development of this methodology presupposes an advance in forensic science. Although a methodology that integrates physical and chemical parameters was recently published [23,29], it has never been possible, until now, to incorporate body weight to improve the estimation, even though its effects on the rectal temperature were known. The elimination of subjective variables in the methodology give greater credibility to the estimation of PMI. Likewise, offering an easy-to-use and free access computer tool with a known marginal error facilitates the estimation of the PMI.

Providing the forensic community with new methodologies for estimating the PMI facilitates their daily routine by offering a choice of validated methods based on the information available in each case.

Although it is true that the statistical approach of this work is not very usual in this field, it is not unknown and it already appears in some works on the IPM, and some in this journal [33–37]

5. Conclusions

The use of VH biochemistry in forensic sciences to estimate the PMI is an objective technique with a known margin of error. Those errors can be minimized by checking if the sample is contaminated, which requires a prior analytical control.

In addition, whichever method is used to estimate the PMI it should incorporate, if possible, those factors that can modify the estimate, thereby indeed minimizing the error in the estimate. Out of all the models designed and tested here, the one that best estimates PMI uses rectal temperature, body weight and the concentrations of hypoxanthine, potassium and urea present in the VH. Quick and easy data measurement makes the application of these models more suitable in forensic practice.

Despite the statistical complexity, obtaining PMI estimation can be performed in an easy, fast and reliable way using software designed for this purpose, which provides the error made in each estimation by the computation of the confidence intervals.

The use of validated and easily reproducible methodologies to estimate the PMI should be imperative in the forensic sciences, given the impact that the results of this type of evidence can have on judicial decisions.

Author contributions

Cordeiro: selecting and performing the autopsies, collecting samples, pretreatment and analysis

Ordóñez Mayán: Data curation, pretreatment, analysis, graphic art

Lendoiro: pretreatment and analysis

Febrero-Bande: Validation, statistical process, software design

Vieira: designing the project, discussing the results

Muñoz-Barús: designing the project, discussing the results and writing – review & editing.

Declarations of interest

None.

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